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Flow Cytometry of Acridine Orange Stained Sperm Is a Rapid and Practical Method for Monitoring Occupational Exposure to Genotoxicants

South Dakota State Univ., Brookings

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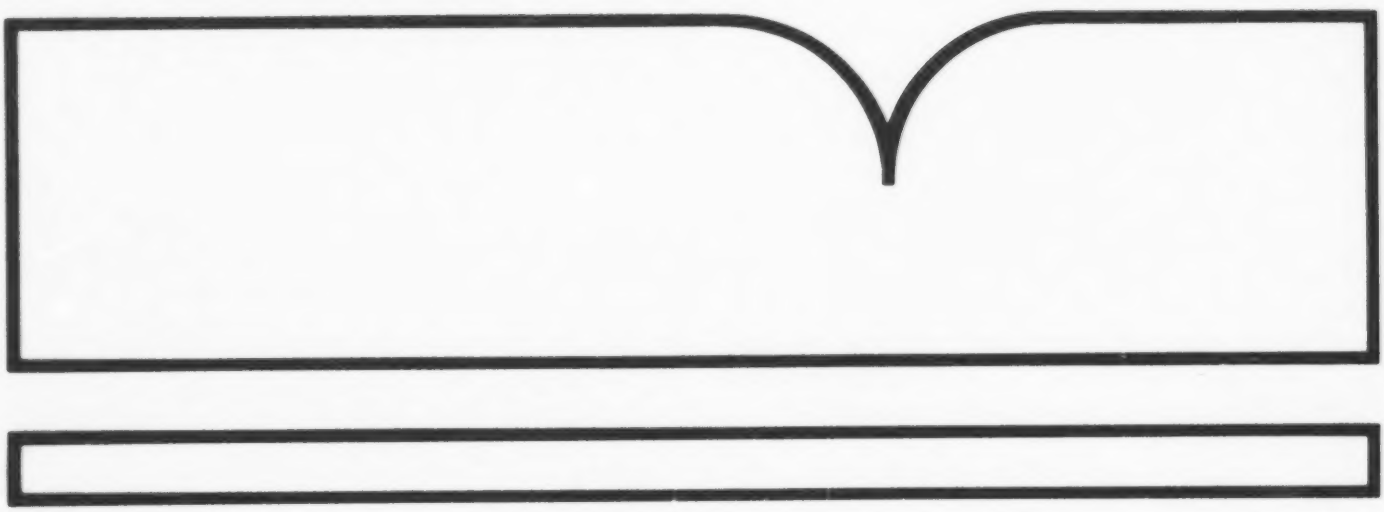

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To detect toxic effects of chemical exposure on germ cells, it is of prime importance to develop more sensitive and practical methods by which putative alterations in germ cells may be investigated. Previously selected (Overstreet 1984) criteria for male reproductive risk assessment required the tests be a) objective, b) technically sound, c) biologically stable, d) sensitive, and e) feasible. Flow cytometric measurement of acridine orange stained sperm meets all of these criteria for monitoring occupational exposure to genotoxicants. This conclusion is based on two independent studies of sperm obtained from: 1) toxin exposed mice, and 2) patients attending infertility and cancer treatment clinics.

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FLOW CYTOMETRY OF ACRIDINE ORANGE STAINED SPERM IS A RAPID AND PRACTICAL METHOD FOR MONITORING OCCUPATIONAL EXPOSURE TO GENOTOXICANTS

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Public awareness is growing concerning the reproductive consequences of the numerous environmental and occupational chemicals. Exposure of germ cells within the seminiferous tubules of the mammalian testis to chemical toxins often causes severe perturbation of cell growth, division and differentiation. Quantitative and qualitative reduction of sperm may have adverse consequences for fertility, and normality of fetus.

To detect toxic effects of chemical exposure on germ cells, it is of prime importance to develop more sensitive and practical methods by which putative alterations in germ cells may be investigated. Previously selected (Overstreet, 1984) criteria for male reproductive risk assessment required the tests be a) objective, b) technically sound, c) biologically stable, d) sensitive, and e) feasible. Flow cytometric measurement of acridine orange stained sperm meets all of these criteria for monitoring occupational exposure to genotoxicants. This conclusion is based on two independent studies of sperm obtained from: 1) toxin exposed mice, and 2) patients attending infertility and cancer treatment clinics.

FLOW CYTOMETRY OF SPERM OBTAINED FROM TOXIN EXPOSED MICE

Previous work (Wyrobek and Bruce, 1975; see Wyrobek et al, 1983 and Topham, 1983 for reviews) has shown that mouse sperm head morphology is sensitive to toxic chemical exposure. These observations led to development of the sperm

head morphology assay which has proven useful for reproductive toxicology because 100% of germ cell mutagens tested have demonstrated a positive response (Wyrobek et al, 1983). This assay is currently performed by light microscope measurements of stained epididymal sperm isolated from toxin-exposed mice. Consequently, the assay suffers from some of the typical limitations of light microscopy, e.g., slowness of measurement, human eye judgments, and often small numbers of observations which limit statistical significance.

Recently we developed (Evenson et al, 1980; 1985a) the flow cytometric sperm chromatin structure assay (SCSA) that appears to be as sensitive as the sperm head morphology assay for detecting toxin-induced abnormalities in mouse sperm nuclei. The SCSA measures the susceptibility to acid- or thermal-induced denaturation of sperm nuclear DNA in situ.

Our experimental flow cytometric approach for the SCSA is illustrated by recent work using ethylnitrosourea (ENU), a powerful alkylating agent. Groups of F_1 male mice (C57BL/6J x C3H/HeJ) were exposed i.p. to phosphate-buffered saline (PBS; control) or dosages of ENU ranging from 5 to 75 mg/kg body weight in PBS, daily x 5 days. Twenty-eight days following the last chemical exposure the mice were killed. The caudal epididymides were excised and placed into TNE (0.01 M TRIS, 0.15 M NaCl, 1 mM EDTA, pH 7.4), minced with scissors and the sperm filtered through 153 um nylon mesh.

Sperm Head Morphology Assay

An aliquot of each epididymal sperm suspension was stained with Eosin Y, smeared onto glass slides and mounted. A minimum of 1000 sperm heads were scored by the morphology criteria of Wyrobek and Bruce (1975). Fig. 1 shows that sperm isolated from ENU exposed mice have a dose-related increase of head morphology abnormalities.

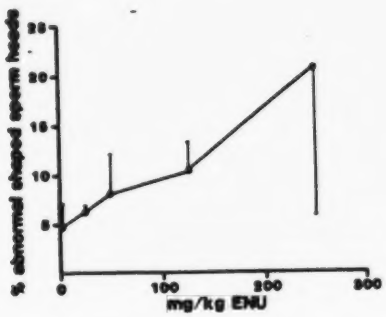

Figure 1. Effects of ENU on caudal epididymal sperm head morphology. Each point represents the mean from three mice; vertical lines show the standard deviations. A repeat experiment demonstrated the same response; however, the standard deviation at the highest dose was much smaller (from Evenson et al, 1985a).

Sperm Chromatin Structure Assay/Acid (SCSA/acid) of Fresh Epididymal Sperm

Epididymal sperm were stained by the TSAO (two step acridine orange) technique (Darzynkiewicz et al, 1976; Evenson et al, 1985a) and measured by flow cytometry. This procedure includes a 30 sec treatment of the sample with Triton X-100, which permealizes the cell membranes for dye penetration, and also 0.06 N HCl which may cause partial denaturation of sperm nuclear DNA in situ. The extent of DNA denaturation was measured by the metachromatic shift in acridine orange (AO) stained sperm from green fluorescence (AO intercalated into double-stranded DNA) to red fluorescence (AO associated with single-stranded DNA). Fig. 2 illustrates the effect of ENU on total fluorescence vs α_t (red/red + green fluorescence) of AO stained epididymal sperm. The term α_t provides a numerical index for the extent of DNA denaturation in situ (Darzynkiewicz et al, 1975). The total fluorescence (green + red, corresponding to double- and single-stranded nucleic acids, respectively) did not increase in response to ENU exposure indicating ENU did not cause an abnormal increase or retention of DNA or RNA. Furthermore, RNAse treatment

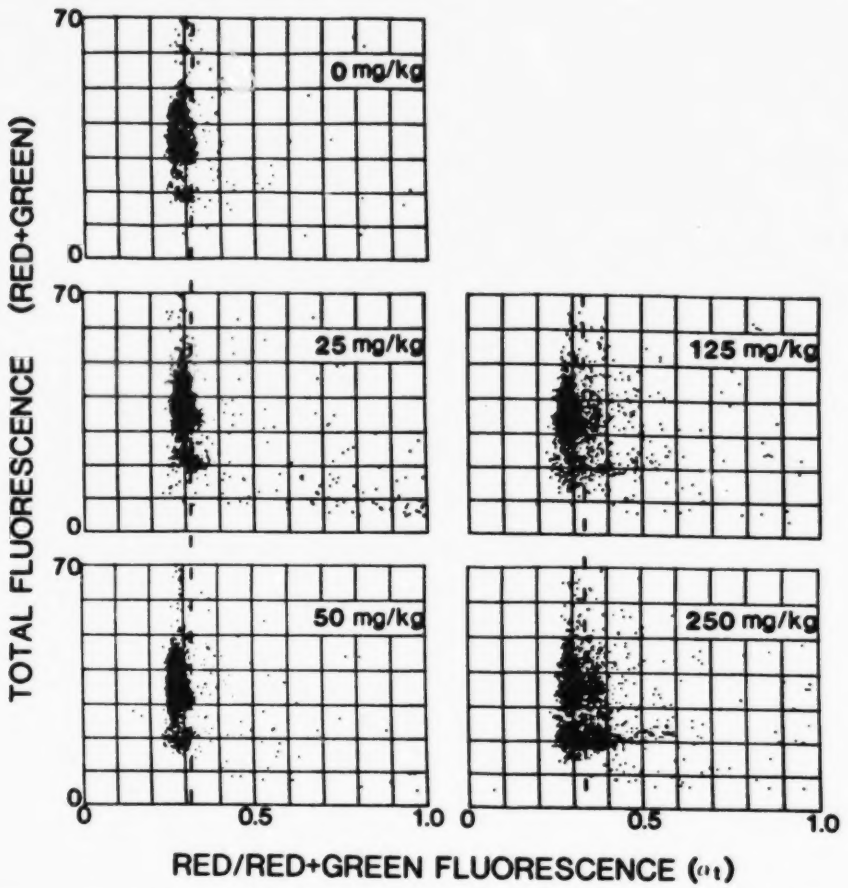

Figure 2. Relationship between exposure level to ENU and red and green fluorescence emission of epididymal sperm analyzed by the SCSA/acid (from Evenson et al, 1985a).

did not alter this staining pattern. Note the shift towards higher α_t values indicating an ENU dose-related alteration of chromatin structure as defined by an increased sensitivity to DNA denaturation.

Sperm Chromatin Structure Assay/Thermal (SCSA/thermal) of Fixed Epididymal Sperm Nuclei

Epididymal sperm nuclei were isolated by sonication

of sperm samples represented in Fig. 2. After fixation (ethanol:acetone) and rehydration, the nuclei were heated at 100°C for 5 min and then stained with AO (Evenson et al, 1980, 1985a). Fig. 3 shows ENU also caused an increased susceptibility to thermal denaturation of DNA <u>in situ</u>.

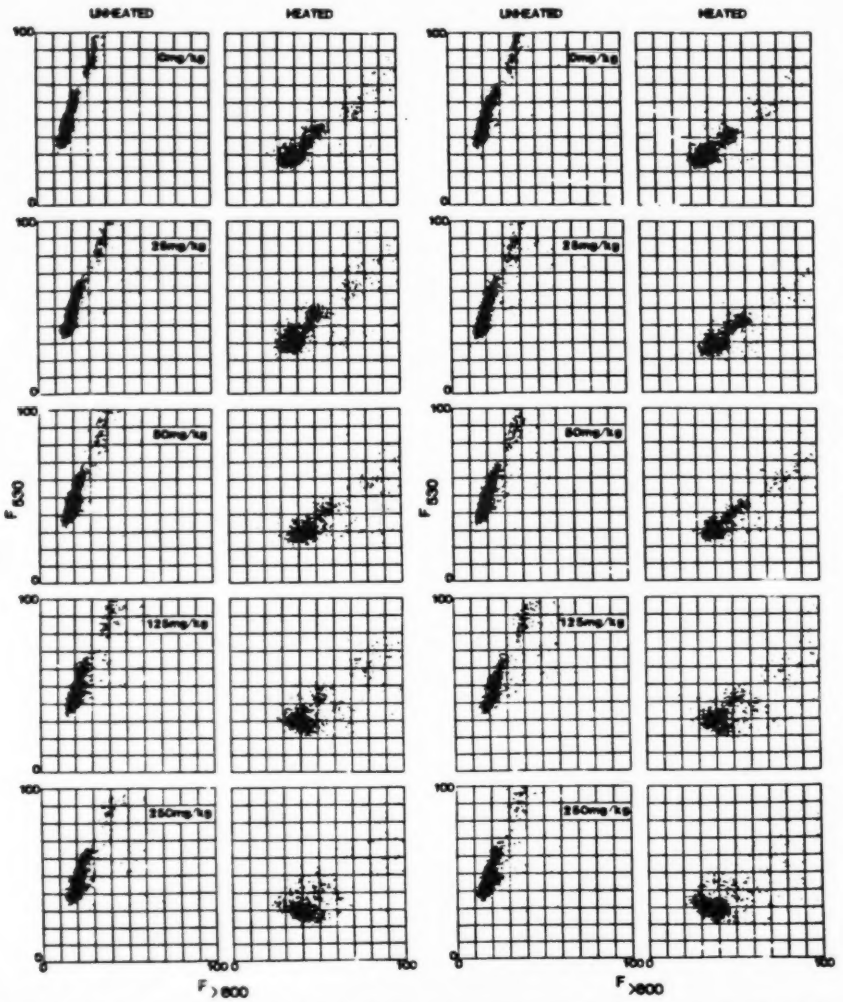

Figure 3. Relationship between exposure level to ENU and red and green fluorescence emission of epididymal sperm nuclei analyzed by the SCSA/thermal. Two independent sets of measurements on heated and non-heated AO stained nuclei were made; note the high level of reproducibility between the two sets (from Evenson et al, 1985a).

Fig. 4 shows computer-plotted α_t frequency histograms of the 5 samples represented in Fig. 3, left-hand 'heated' column. Note the increased shoulder seen even at the lowest dosage (25 mg/kg).

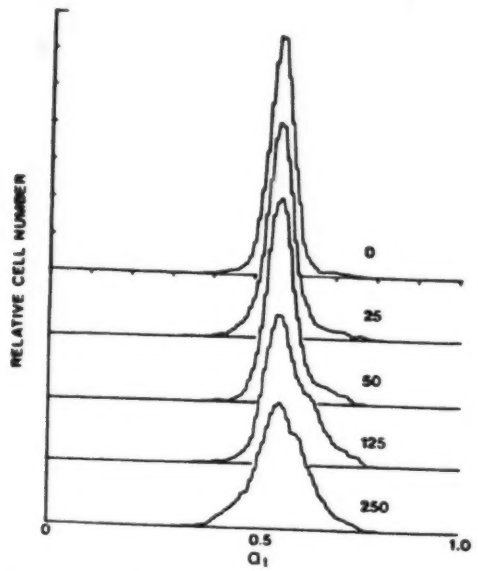

Figure 4. Effect of ENU on mouse sperm α_t distributions (from Evenson et al, 1985a).

It is obvious from the data in Fig. 4 that the coefficient of variation (C.V.) of α_t increases with dosage of ENU exposure; this relationship is plotted in Fig. 5.

Interestingly, the curve in Fig. 5 is virtually identical to the C.V. α_t of whole sperm evaluated by the SCSA/acid ($r = 0.9$; data not shown). Apparently both the acid and heat treatment prior to AO staining resolve the same toxin-induced abnormality of chromatin structure.

Comparison of data in Fig. 1 with that in Fig. 5 shows that the flow cytometric-based SCSA is as sensitive as the sperm head morphology assay. Further work on other chemicals including thiotepa, metepa, caffeine, actinomycin D, hydroxyurea, methylmethanesulfonate, ethylmethane-

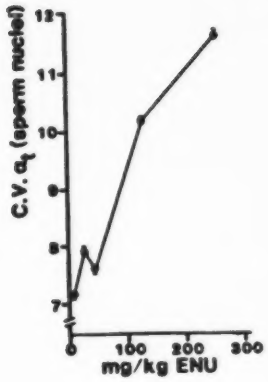

Figure 5. Relationship between exposure level to ENU and coefficient of variation of α_t of epididymal sperm nuclei evaluated by the SCSA/thermal (from Evenson et al, 1985a).

sulfonate and others (Evenson et al, 1985c) confirms this point. Regression analyses showed, with one minor exception, the slopes of the curves of sperm morphology abnormalities and standard deviation of α_t vs chemical dosage were not different from each other. Thus we conclude that our SCSA is measuring a factor of chromatin structure that is highly related to sperm head morphology abnormalities.

FCM MEASUREMENTS OF AO STAINED HUMAN SEMEN SAMPLES

Toxin exposure can cause immature germ cells to be released into semen. Fig. 6 shows FCM derived data on fresh TSAO stained semen aliquots obtained from a normal volunteer and two cancer patients exposed (or not) to chemotherapeutic drugs. The TSAO procedure apparently does not denature histone-associated DNA <u>in situ</u>. Flow cytometer photomultiplier gains were set to include cell types ranging in DNA stainability from sperm to diploid cells. This difference is 10-fold since the peak of the normal sperm population is at channel 8 and the diploid cells peak at channel 80 (patient B). The control sample has a homogeneous distribution with minimal green and red fluorescence. Patient A had

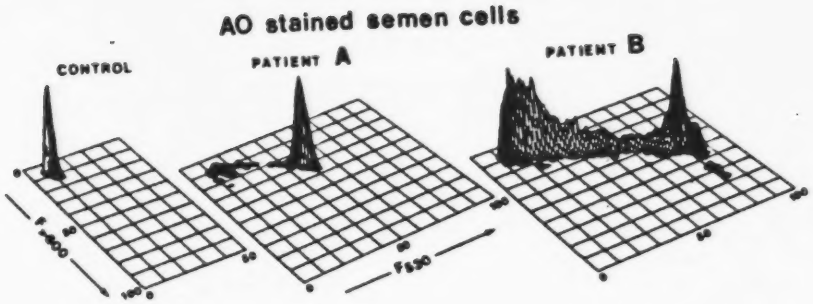

Figure 6. Computer drawn two-parameter (F_{530}, green fluorescence vs $F_{>600}$, red fluorescence) histograms representing distribution of TSAO stained human semen cells A. Normal volunteer, B. Cancer patient exposed to chemotherapy, C. Testicular cancer patient treated by unilateral orchiectomy (from Evenson and Melamed, 1983).

been exposed to chemotherapy drugs 3 months prior to semen sample collection. The sperm count was 5×10^4/ml. Note that these sperm had a higher level of DNA stainability (between channels 10 - 30) indicating a lack of normal chromatin condensation. The majority of cells present in the semen were round spermatids verified by their identical staining pattern to the round spermatids in minced testicular biopsy samples. By light microscopy, it is difficult to distinguish round spermatids from leukocytes which could be present from infections. FCM measurements easily and positively distinguish between these two cell types.

Patient B with testicular cancer had unilateral orchiectomy 11 months prior to collection of a semen sample thus showing that disease alone apparently caused a deterioration of spermatogenesis. In this sample, cells ranging in maturity from diploid cells to nearly mature sperm (but with abnormal morphology) were present.

The majority of patients at infertility clinics do not show such gross semen abnormalities as represented in Fig. 6. To detect lessor levels of chromatin abnormalities, sperm nuclei have been isolated, heated, stained with AO and

measured by FCM according to the SCSA/thermal described above for the mouse studies. Fig. 7 shows data on semen from a fertile donor and 5 patients attending an infertility clinic. Note the dramatic heterogeneity between samples of susceptibility to DNA denaturation.

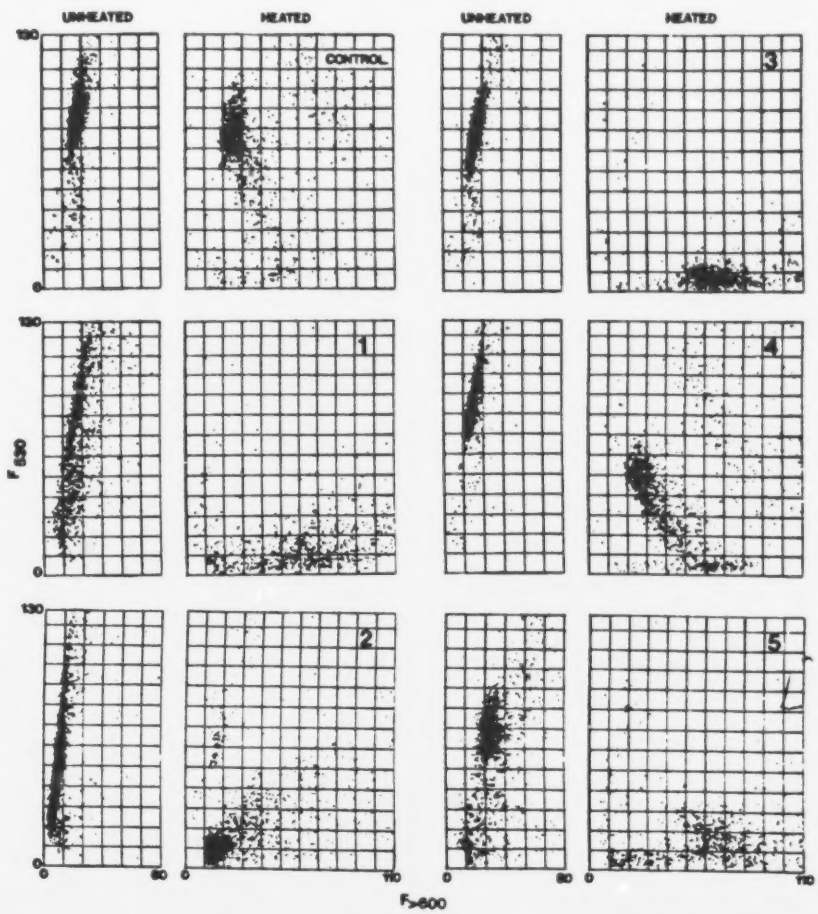

Figure 7. Computer drawn scattergrams of the distribution of sperm nuclei from one control (fertile donor) and 5 patients attending an infertility clinic, according to their green (F_{530}) and red ($F_{>600}$) fluorescence intensities after heating (or not heating) and staining with AO (SCSA/thermal). Patients were diagnosed as follows: (1) Morphology poor to borderline; Infection (I^+); (2) Prostatitis/Non-specific urethritis (NSU), morphology

minimal, I^-; (3) Prostatitis/NSU, good specimen, I^+; (4) Morphology and motility good, 2 miscarriages, I^-; (5) Excellent specimen in all respects (from Evenson et al, 1985b).

Perhaps the most interesting samples are those that are scored as 'excellent' by the classical criteria of sperm count, morphology, and motility but have a significant abnormality of chromatin structure (e.g. patient 5, Fig. 7). Thus, our technique will not only rapidly screen for toxin-induced or other abnormalities present in semen but will detect abnormalities not found by other techniques. Similar FCM measurements have been made on semen samples obtained from testicular carcinoma patients (Evenson et al, 1984a) and leukemia patients successfully treated by chemotherapy (Evenson et al, 1984b).

Flow cytometric measurement of AO stained semen cells or epididymal sperm provides a new, sensitive, objective, biologically stable, technically sound and feasible method to monitor for genotoxicants that may be found in occupational environments. Since semen samples may be collected, frozen and sent to an FCM laboratory for measurements, this method is indeed practical for screening programs.

ACKNOWLEDGEMENTS

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